

# Androgens modulate glucocorticoid receptor activity in adipose tissue and liver

Dieuwertje C.E. Spaanderman<sup>1,2</sup>, Mark Nixon<sup>3</sup>, Jacobus C. Buurstedde<sup>1,2</sup>, Hetty H.C.M. Sips<sup>1,2</sup>, Maaïke Schilperoort<sup>1,2</sup>, Eline N. Kuipers<sup>1,2</sup>, Emma A. Backer<sup>1,2</sup>, Sander Kooijman<sup>1,2</sup>, Patrick C. N. Rensen<sup>1,2</sup>, Natalie Z. M. Homer<sup>3</sup>, Brian R. Walker<sup>3,4</sup>, Onno C. Meijer<sup>1,2,\*</sup>, Jan Kroon<sup>1,2,\*</sup>

<sup>1</sup> Department of Medicine, Division of Endocrinology, Leiden University Medical Center, Leiden, the Netherlands; <sup>2</sup> Eindhoven laboratory for Experimental Vascular Medicine, Leiden University Medical Center, Leiden, the Netherlands; <sup>3</sup> BHF Centre for Cardiovascular Science, Queen's Medical Research Institute, University of Edinburgh, Edinburgh, United Kingdom; <sup>4</sup> Institute for Genetic Medicine, Newcastle University, Newcastle upon Tyne, United Kingdom

\* Shared last author.

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Corresponding author:

Jan Kroon, PhD  
Department of Medicine, Division of Endocrinology  
Leiden University Medical Center  
Albinusdreef 2  
Room C7-44g  
2333 ZA, Leiden, the Netherlands  
Phone: +31-71-5268187  
Email: [j.kroon@lumc.nl](mailto:j.kroon@lumc.nl)

**Abstract**

Glucocorticoid signaling is context-dependent, and in certain scenarios glucocorticoid receptors (GR) are able to engage with other members of the nuclear receptor subfamily. Glucocorticoid signaling can exert sexually dimorphic effects, suggesting a possible interaction with androgen sex hormones. We therefore set out to determine the crosstalk between glucocorticoids and androgens in metabolic tissues including white adipose tissue, liver and brown adipose tissue. Thereto we exposed male C57BL/6J mice to elevated levels of corticosterone in combination with an androgen receptor (AR) agonist or an AR antagonist. Systemic and local glucocorticoid levels were determined by mass spectrometry, tissue expression of glucocorticoid-responsive genes and protein was measured by RT-qPCR and Western blot, respectively. To evaluate crosstalk *in vitro*, cultured white and brown adipocytes were exposed to a combination of corticosterone and an androgen agonist. We found that AR agonism potentiated transcriptional response to GR *in vitro* in white and brown adipocytes and *in vivo* in white and brown adipose tissue. Conversely, AR antagonism substantially attenuated glucocorticoid signaling in white adipose tissue and liver. In white adipose tissue this effect could partially be attributed to decreased 11 $\beta$ -hydroxysteroid dehydrogenase type 1-mediated glucocorticoid regeneration upon AR antagonism. In liver, attenuated GR activity was independent of active glucocorticoid ligand levels. We conclude that androgen signaling modulates GR transcriptional output in a tissue-specific manner.

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## 49 Introduction

50 Glucocorticoids are adrenal steroid hormones engaged in a myriad of functions including adaptation  
51 to stress, circadian function and immune regulation. Glucocorticoids also mediate quintessential  
52 processes in metabolic physiology to ensure whole-body energy homeostasis e.g. by stimulating  
53 hepatic gluconeogenesis, reducing peripheral glucose uptake and inducing adipose tissue lipolysis  
54 (Kadmiel and Cidlowski 2013). Due to the pleiotropic nature of glucocorticoid signaling,  
55 dysregulation inevitably endangers metabolic health. This is seen in Cushing's syndrome, where  
56 glucocorticoid excess causes massive accumulation and redistribution of energy stores, accompanied  
57 by cardiometabolic harm (Morgan, et al. 2016).

58 Glucocorticoid action in metabolism is predominantly mediated by glucocorticoid receptors (GR)  
59 which are expressed in virtually all tissues in both humans and rodents. It is known that  
60 glucocorticoid transcriptional activity is subject to multiple levels of regulation; from the production,  
61 secretion and tissue uptake/export of steroid ligands to receptor expression and DNA-binding.  
62 Intracellular enzymatic (in)activation controls glucocorticoid activity, i.e. 11 $\beta$ -hydroxysteroid  
63 dehydrogenase type 1 (11 $\beta$ -HSD1) catalyzes glucocorticoid regeneration from inactive steroid while  
64 11 $\beta$ -hydroxysteroid dehydrogenase type 2 (11 $\beta$ -HSD2) inactivates the active ligand pool. Active  
65 ligand binding to GR provokes its translocation from the cytoplasm to the nucleus. GR binds DNA at  
66 so-called glucocorticoid response elements thereby facilitating gene induction or repression  
67 (Ratman, et al. 2013).

68 The extent of GR-regulated transcriptional activity is dictated by ligand availability, receptor  
69 expression and the DNA-binding site (Meijsing, et al. 2009), but also by interactions with  
70 coregulatory proteins and other (often unknown) signaling partners (Meijer, et al. 2005).

71 Glucocorticoids therefore act in a context-dependent manner and it was previously shown that  
72 glucocorticoids can have sexually dimorphic actions (Duma, et al. 2010; Quinn and Cidlowski 2016).

73 For example, in the liver the synthetic glucocorticoid dexamethasone differentially affects expression

74 of many GR target genes in male vs. female rats (Duma et al. 2010). Such sexually dimorphic effects  
75 could possibly be explained by glucocorticoid interactions with sex hormone signaling, e.g. androgen  
76 receptor (AR) activity. AR signaling is known to regulate physiological processes like adipose tissue  
77 lipolysis and hepatic glucose and lipid metabolism (Lee, et al. 2013; Shen and Shi 2015), but it is yet  
78 unclear if AR signaling can contribute to the sexually dimorphic effects of GR.

79 In certain contexts, GRs are able to engage with other members of the steroid nuclear receptor  
80 subfamily (Chen, et al. 1997; Mifsud and Reul 2016). All six nuclear steroid receptors, including the  
81 GR and the AR, originate from a common ancestral receptor gene (Thornton 2001), which underlies  
82 the substantial homology in DNA-binding domain and hormone-response elements. For example in  
83 cancer, the chromatin binding landscape of the GR is exceptionally similar to that of other nuclear  
84 steroid receptors (Severson, et al. 2018), GR shares numerous DNA binding sites with the AR (Arora,  
85 et al. 2013), and these two receptors may even form heterodimers on glucocorticoid response  
86 elements (Chen et al. 1997). However, beyond cancer the crosstalk between GR and AR has  
87 remained elusive.

88 In this study, we explored if crosstalk between androgens and glucocorticoids exists in metabolic  
89 tissues. In male C57BL/6J mice, we investigated possible interactions in white adipose tissue (WAT),  
90 liver, and brown adipose tissue (BAT) - all metabolically-active tissues that contribute considerably to  
91 whole-body metabolism. We reveal that androgen signaling strongly influences metabolic GR  
92 signaling, and that the underlying mechanisms are highly tissue-specific.

93

## 94 **Material and methods**

### 95 **Animals**

96 All animal studies were approved by the ethical committee of Leiden University Medical Center.  
97 Mice were housed in conventional cages with a 12:12-hour light-dark cycle and *ad libitum* access to

food and water. To examine the effect of AR signaling on metabolic glucocorticoid action, 8-week old male C57BL6/J mice (Jackson Laboratory) were exposed to the GR agonist corticosterone (CORT; Sigma-Aldrich) and AR agonist dihydrotestosterone (DHT; 5 $\alpha$ -Androstan-17 $\beta$ -ol-3-one, Sigma-Aldrich) via subcutaneous implantation of slow-release pellets (100 mg). The AR antagonist Enzalutamide (MedChemExpress) was administered via diet-supplementation (357 mg drug per kg diet; resulting in an estimated dose of 40 mg/kg/day). The following experimental groups were followed for a period of 14 days: 1) Vehicle (N=6), 2) CORT (20 mg corticosterone; N=7), 3) CORT+DHT (20 mg corticosterone, 30 mg DHT; N=7) and 4) CORT+Enza (20 mg corticosterone + Enzalutamide-treatment; N=7). At baseline and after 14 days, body weight was measured with a scale and body composition was determined by echo-MRI. After seven days of intervention, mice were fasted for 6 hours and blood was collected for analysis of plasma triglycerides, cholesterol and free fatty acids. After 14 days mice were euthanized by CO<sub>2</sub> inhalation, blood was drawn via heart puncture in EDTA-coated tubes, mice were perfused with ice-cold PBS for 5 min and metabolic organs (i.e. gonadal and subcutaneous WAT, liver, interscapular BAT) were collected for molecular, functional and histological analyses.

To evaluate the effect of Enzalutamide alone, we performed a control experiment in which mice were treated with vehicle (N=6) or Enzalutamide (40 mg/kg/day via diet; N=6) for 27 days. At baseline and after 27 days, body weight was measured with a scale and body composition was determined by echo-MRI. Blood was collected at day 21 after a 6 hour fast, for analysis of plasma triglycerides, cholesterol and free fatty acids. After 27 days, mice were euthanized by CO<sub>2</sub> inhalation, perfused with ice-cold PBS for 5 min and WAT and liver were collected for molecular analysis.

#### **RNA isolation, cDNA synthesis and real-time quantitative (RT)-qPCR analysis**

Snap-frozen tissue samples of gonadal WAT, liver and BAT were homogenized in Tripure (Roche) and mRNA was isolated according to the manufacturer's protocol. 500-1000 ng mRNA was used for cDNA

synthesis using M-MLV reverse transcriptase (Promega), and gene expression was assessed with RT-qPCR. Primer sequences are available upon request.

#### **Western blot**

Western blot was performed using the Wes apparatus (ProteinSimple), according to the manufacturer's protocol. For WAT and liver samples, 2.0 and 0.8 mg/ml lysate was used, respectively. The Wes apparatus allows simultaneous detection of multiple proteins and therefore loading controls may be identical between different figures. The following primary and secondary antibodies were used: 11BHSD1 (in house antibody University of Edinburgh, 1:10), FKBP5 (AB\_2103136, R&D systems, AF4094, 1:70), GAPDH (AB\_10167668, Santa Cruz Biotechnology, sc25778, 1:50), GR (AB\_2631286, Cell signaling, 12401, 1:10), HRP anti-goat (ProteinSimple, DM006), HRP anti-rabbit (ProteinSimple, DM001) and HRP anti-sheep (AB\_955452, Abcam, ab6900, 1:100).

#### **Steroid extraction and liquid chromatography-mass spectrometry (LC-MS) analysis**

Steroids were extracted from plasma (100 µl, obtained from heart puncture) and snap-frozen gonadal WAT and liver tissues (approx. 200 mg) and were subsequently analyzed using LC-MS. Plasma samples were diluted 1:1 with ammonium hydroxide and enriched with internal standards epicorticoosterone, D4-cortisol, <sup>13</sup>C3-testosterone and <sup>13</sup>C3-dihydrotestosterone. Plasma samples were applied to SLE200+ columns, eluted with 95:5 DCM:isopropanol, dried down under hydrogen and reconstituted in 70 µl mobile phase (70:30 H<sub>2</sub>O:CH<sub>3</sub>CN). Liver and WAT samples were homogenized in respectively 7:2 MeOH:H<sub>2</sub>O and ethyl acetate, and were enriched with internal standards epicorticoosterone and D4-cortisol. The liver homogenates were dried down under nitrogen and dissolved in 30% MeOH. The WAT homogenates were dripped into chilled EtOH:Acetic acid:H<sub>2</sub>O, sonicated for 8x15 seconds, dried down under nitrogen and reconstituted in 1:1 MeOH:hexane. After inversion the MeOH layer was dried down and reconstituted in 10:1 ethyl acetate:H<sub>2</sub>O. The ethyl acetate layer was isolated and dried down after which it was resuspended in

30% MeOH. Both liver and WAT samples were applied to bond elute C18 columns (Agilent Technologies). The steroid-containing elutes were dried down and dissolved in 70 µl mobile phase (70:30 H<sub>2</sub>O:acetonitrile) for simultaneous mass spectrometry measurement of the glucocorticoids CORT and 11-dehydrocorticosterone (11-DHC), and the androgens testosterone and DHT.

## Cell culture

Human HEK293T cells were passaged in culture medium (DMEM+Glutamax supplemented with 10% FCS and penicillin/streptomycin) and during experiments medium with charcoal-stripped serum was used. Cells were transfected using Eugene HD (Promega) with 25 ng TAT3-luciferase, 10 ng human AR or GR, 1 ng CAGGS-renilla and 100 ng pcDNA. Cells were pretreated with Enzalutamide for 30 min before agonists for AR (Testosterone or DHT) or GR (cortisol) were added. After 24 hours, firefly- and renilla-luciferase signals were measured using a dual-luciferase assay (Promega).

Murine 3T3.L1 cells were passaged in culture medium (DMEM+Glutamax supplemented with 10% FCS and penicillin/streptomycin). For experiments, preadipocytes were seeded and medium was replaced every 2-3 days. After reaching confluence, adipogenic differentiation was induced by adding medium supplemented with 250 nM dexamethasone, 11 mg/ml IBMX and 10 mg/ml insulin for 2 days, medium supplemented with 10 mg/mL insulin for 6 days and normal culture medium for 7 days. During the last 2 days of differentiation and during treatments FCS was replaced with charcoal-stripped serum. Cells were pretreated with 100 nM DHT and after 1 hour 10-30 nM corticosterone was added for 24 hours. Cells were harvested in Tripure for further RT-PCR analysis of GR-responsive transcripts. Murine immortalized brown adipocytes were generated and cultured as previously published (Kroon, et al. 2018) and exposure with DHT and corticosterone was identical as in 3T3.L1 cells.

## Statistical analysis

All data are presented as mean  $\pm$  SEM. Statistical analysis were performed with GraphPad Prism 7 software (GraphPad Inc.). To calculate statistical differences between more than two groups, a one-way analysis of variance with Tukey multiple-comparison test was used. For direct comparison of two groups, an unpaired students t-test was performed.

## Results

### Confirmation of glucocorticoid and androgen interventions

We set out to investigate the crosstalk between glucocorticoid and androgen signaling in metabolic tissues. AR agonist DHT and AR antagonist Enzalutamide were used, for which reporter assays in HEK293T cells confirmed effective AR agonism and antagonism, respectively (**Fig. 1A-B**). Enzalutamide did not exhibit GR cross-reactivity (**Fig. 1C**) (Dalal, et al. 2014). To investigate crosstalk *in vivo*, male C57BL/6J mice were implanted with CORT-releasing pellets (van den Beukel, et al. 2015) in combination with DHT or Enzalutamide. Effective increased glucocorticoid exposure was confirmed by decreased adrenal weight (biomarker for ACTH stimulation of the adrenal cortex; **Fig. 1D**). DHT and Enzalutamide interventions were confirmed by increased and decreased weight of the androgen-responsive seminal vesicles, respectively (**Fig. 1E**). In addition enhanced plasma and tissue levels of DHT in the DHT group (**Fig. 1F-H**) and increased plasma levels of testosterone upon Enzalutamide treatment (due to relieved negative feedback on testosterone secretion) confirm the efficacy of experimental interventions (**Fig. 1I-K**).

### AR antagonism lowers glucocorticoid-induced hyperlipidemia

Elevated CORT did not influence total body weight but did alter body composition (seemingly enhanced fat mass and significantly reduced lean mass; **Fig. 2A**) and induced hyperlipidemia (**Fig.**



2B). DHT generally did not affect CORT-induced metabolic outcome, with the exception of lean body mass (Fig. 2A). Notably, concomitant AR antagonism with Enzalutamide significantly lowered 6 hour-fasted plasma levels of triglycerides ( $p < 0.01$  vs CORT), cholesterol ( $p < 0.01$  vs CORT) and free fatty acids ( $p < 0.05$  vs CORT), thus alleviating CORT-induced dyslipidemia (Fig. 2B). Treatment with Enzalutamide alone did not alter body weight and composition (Fig. 2C), but did lower plasma lipid levels (Fig. 2D).

#### AR antagonism attenuates glucocorticoid-induced transcriptional activity in a tissue-specific manner

GR-regulated transcriptional activity was evaluated in multiple metabolic tissues by RT-PCR analysis of well-established glucocorticoid-responsive transcripts *FK506 binding protein 5 (Fkbp5)*, *TSC22D3 (Gilz)* and *Metallothionein-II (Mt2a)* (Arora et al. 2013; Bolton, et al. 2007; Wilson, et al. 2016). As expected CORT upregulated expression of GR-responsive genes in WAT (Fig. 3A-C), liver (Fig. 3D-F) and BAT (Fig. 3G-I). Compared to CORT alone, AR agonist DHT further enhanced expression of WAT *Fkbp5* ( $p < 0.09$ , Fig. 3A) and *Mt2a* ( $p < 0.05$ , Fig. 3C), and BAT *Fkbp5* ( $p < 0.01$ , Fig. 3G) and *Gilz* ( $p < 0.01$ , Fig. 3H). Interestingly, AR antagonism with Enzalutamide diminished CORT-induced GR transcriptional activity in WAT and liver (Fig. 3A-F), but did not influence GR transcriptional activity in BAT (Fig. 3G-I). Also at the protein level, CORT-induced FKBP5 content in both WAT and liver was attenuated with AR antagonism (Fig. 3J-K). Enzalutamide treatment alone did not alter GR-responsive genes in WAT and liver (with the possible exception of *Mt2a* in liver; Fig. 3M-N), confirming that AR antagonism specifically attenuates CORT-induced GR activity. In addition to classic GR target genes, we interrogated the expression of genes involved in metabolic function: lipolysis genes *hormone-sensitive lipase (Hsl)* and *adipocyte triglyceride lipase (Atgl)* were increased after combined CORT and DHT exposure in WAT; very-low-density-lipoprotein production genes *microsomal triglyceride transfer protein (Mttp)* and *Apolipoprotein B (Apob)* in the liver were induced by CORT and attenuated by concurrent AR antagonism; while thermogenic gene *Ucp1* in BAT was

unaffected by all interventions (**Fig. 3L**). Our data highlight the tissue-specific nature of glucocorticoid-androgen interactions and a possible effect on metabolic pathways.

#### **AR agonism potentiates GR signaling in fully differentiated murine (brown) adipocytes**

To further investigate the crosstalk between androgens and glucocorticoids directly on adipocytes - we utilized cell-based culture models of immortalized white and brown adipocytes. We first confirmed *Nr3c1* and *Nr3c4* expression in fully differentiated white and brown adipocytes (**Fig. 4A-B**). Next, we determined the expression of GR-responsive genes after 24 hour exposure to CORT with and without a 1 hour pretreatment with DHT. CORT treatment alone resulted in a dose-dependent induction of *Fkbp5*, *Gilz*, *Mt2a* and *Atgl* in 3T3.L1 white adipocyte cells (**Fig. 4C-F**) and *Fkbp5* in immortalized brown adipocytes (**Fig. 4G**). DHT on its own (100 nM) did not affect transcript levels, with the exception of *Fkbp5* expression in brown adipocytes which was marginally upregulated (1.4-fold induction,  $p<0.05$ , **Fig. 4G**). Supporting our *in vivo* data, combined exposure to CORT and DHT seemed to synergistically upregulate expression of *Fkbp5* ( $p<0.05$  at 30 nM CORT; **Fig. 4C**), *Gilz* ( $p<0.001$  at 10 nM CORT; **Fig. 4C**) and *Mt2a* ( $p<0.05$  at 10 nM CORT; **Fig. 4E**) in 3T3.L1 cells. CORT-induced expression of lipolysis gene *Atgl* was not significantly increased upon DHT treatment (**Fig. 4F**). In brown adipocytes we observed a similar potentiation for expression of *Fkbp5* (**Fig. 4G**) and possibly *Gilz* (**Fig. 4H**) but not for *Mt2a* and *Ucp1* (**Fig. 4I-J**), supporting our observations *in vivo* in BAT. Altogether we observe interactions between glucocorticoid and androgen signaling *in vitro*, as androgens exposure potentiates GR activity at physiological glucocorticoid concentrations.

#### **AR antagonism tissue-specifically alters corticosterone and 11-dehydrocorticosterone levels**

We attempted to decipher how AR signaling alters GR-regulated transcriptional activity. GR expression levels in WAT and liver were not causally involved: CORT downregulated GR protein expression via homologous downregulation (Dong, et al. 1988), but androgen interventions did not further affect GR expression (**Fig 5A-D**). To analyze if glucocorticoid turnover is affected by androgen

signaling we determined tissue-specific levels of ligand CORT and its inactive metabolite 11-DHC. At equal systemic CORT exposure (**Fig. 6A**), AR antagonism significantly decreased CORT levels in WAT ( $p < 0.01$ , **Fig. 6B**), while this was unaltered by AR agonism. In contrast to WAT, AR antagonism did not influence hepatic CORT levels (**Fig. 6C**). Plasma and liver levels of the inactive glucocorticoid metabolite 11-DHC were significantly increased upon AR blockade (**Fig. 6D, F**), but were not changed in WAT (**Fig. 6E**). Androgen signaling thus seems to modulate glucocorticoid turnover, as the CORT/11-DHC ratio in plasma, WAT and liver was consistently lower upon AR antagonism (**Fig. 6G-I**), influencing tissue-specific glucocorticoid ligand levels.

#### **AR antagonism decreases 11BHSD1 expression which underlies GR transcriptional activity in WAT but not liver**

The balance between active vs inactive glucocorticoids in the circulation is predominantly dictated by peripheral and central 11BHSD1 activity and renal 11BHSD2 activity (Pereira, et al. 2012). Local active glucocorticoid levels may however be skewed by tissue-specific 11BHSD1 activity, and to explore if this contributes to altered glucocorticoid turnover upon AR blockage we analyzed mRNA and protein expression of 11BHSD1. CORT upregulated *hsd11b1* mRNA expression in WAT and liver (**Fig. 7A-B**). In WAT, this upregulation was accompanied by increased 11BHSD1 protein expression (**Fig. 7C**). Notably, this upregulation was abolished by AR blockade ( $p < 0.05$  for protein; **Fig. 7C**), suggesting that AR signaling influences glucocorticoid turnover in WAT. In liver, AR antagonism attenuated CORT-induced *hsd11b1* mRNA expression (**Fig. 7B**), but protein expression did not follow this pattern (**Fig. 7D**). We next analyzed if altered glucocorticoid turnover contributed to the reduced GR-regulated transcriptional activity upon AR antagonism. Thereto we plotted tissue-specific CORT concentrations against GR-responsive transcript expression, revealing that *Fkbp5*, *Gilz* and *Mt2a* expression proportionally changed in parallel with ligand concentrations in WAT (**Fig. 7E**). In contrast to WAT, GR-regulated transcriptional activity in liver was altered independent of tissue CORT

concentration (**Fig. 7F**). Altered 11BHSD1-mediated glucocorticoid turnover thus seems to partially underlie diminished GR-regulated transcriptional activity in WAT but not in liver.

## Discussion

In this study we set out to investigate if crosstalk between androgens and glucocorticoids exists in metabolic tissues. While glucocorticoid-androgen interactions are widely described in prostate cancer (Arora et al. 2013; Li, et al. 2017; Puhr, et al. 2018; Xie, et al. 2015), the importance for metabolic tissues has yet remained elusive. This and previously published findings that glucocorticoids can act in a sexually dimorphic manner (Duma et al. 2010; Quinn and Cidlowski 2016) prompted us to investigate the potential modulating role of androgen sex hormones in metabolic glucocorticoid signaling. In our Cushing's-like model for excessive glucocorticoid exposure, with metabolic features comparable to human Cushing's syndrome (Ferrau and Korbonits 2015), we observed distinct patterns of glucocorticoid-androgen crosstalk in different metabolic tissues: i.e. AR-mediated potentiation of GR signaling in WAT and BAT, and attenuated GR signaling upon AR blockade in WAT and liver.

Concerning the increased GR-target gene expression upon simultaneous CORT and DHT exposure, it has to be noted that the enhanced GR-target gene expression observed upon DHT treatment may either be the consequence of AR-mediated potentiation of GR signaling or it could be an additive effect, reflecting the promiscuous nature of many GR-responsive transcripts that can also be AR-responsive. Our findings in cultured white and brown adipocytes show that DHT alone does not induce *Gilz* and *Mt2* expression, but that combined with CORT it synergistically enhances transcription. This suggests that a potentiation of GR signaling by AR is responsible for the increased transcription, rather than additive transcriptional effects of both receptors on shared GR-AR target genes. On the other hand, *Fkbp5* expression was slightly induced by DHT alone. Part of the effect

could thus be mediated via direct AR-mediated transcription on this shared GR-AR target gene, possibly independent of GR (Arora et al. 2013). Nevertheless, *Fkbp5* induction upon combined CORT and DHT exceeds the effect observed upon DHT alone, thus also pointing to potentiation of GR-mediated *Fkbp5* transcription by androgens. Aside from enhanced transcription upon DHT, we found that AR antagonism attenuates target gene expression and AR is thus required for effective GR-regulated transcriptional activity. This could be a direct effect, i.e. physical presence of AR is essential to facilitate GR activity, or an indirect effect, i.e. expression of factors that modulate GR activity are AR-driven. Enzalutamide treatment could reduce expression of AR-regulated factors that are required for adequate GR activity. Since Enzalutamide prevents nuclear translocation of AR (Schalken and Fitzpatrick 2016), this argues against active physical repression of transcription by Enzalutamide-bound AR on shared GR-AR loci.

Although glucocorticoids can have pleiotropic effects on WAT by stimulating both lipolysis and lipogenesis (Lee, et al. 2014), they are classically perceived as catabolic hormones (e.g. stimulating protein breakdown in skeletal muscle and decreasing bone formation) (Cohen and Steger 2017). In contrast, androgens are mainly anabolic (e.g. promoting muscle growth, formation of trabecular and cortical bone mass). Despite these seemingly opposite functions, our data suggest that both classes of steroids also have the capacity to cooperate in certain tissues. This convergence of signaling may initially support a common goal - setting free energy substrates by e.g. lipolysis (Lee et al. 2013; Peckett, et al. 2011) – but subsequently aids differential functions: energy mobilization to cope with a stressful situation for glucocorticoids and generating fuel for anabolic growth for androgens. In our studies, we indeed observe collaborative signaling between glucocorticoids and androgens in WAT – which has lipolytic capacity (Wang, et al. 2015) - but how glucocorticoid-androgen interplay affects fuel-utilizing tissues is a subject for further study.

Our data indicate that androgens modulate glucocorticoid signaling via parallel, non-mutually exclusive mechanisms. We show that androgen signaling influences activity of 11BHSD isozymes,

resulting in altered systemic and tissue-specific glucocorticoid turnover. Systemically, AR antagonism increased inert glucocorticoid levels, likely due to decreased peripheral 11BHSD1 activity. It has been described that 11BHSD1-deficient tissues are shielded from glucocorticoid effects (Doig, et al. 2017; Morgan, et al. 2014). AR antagonism mimics this effect in WAT, as decreased CORT levels were found due to impaired local glucocorticoid regeneration. The decreased levels of active glucocorticoids were associated with diminished GR-regulated transcriptional activity in WAT. In liver a discrepancy was found between 11BHSD1 mRNA and protein expression, with reduced GR-regulated transcriptional activity upon AR antagonism shown to be independent of glucocorticoid ligand concentration. Differential 11BHSD1 activity upon AR antagonism does thus not underlie blunted hepatic GR response. Possibly, crosstalk between GR and AR prevails at the DNA-binding level. In different peripheral tissues, distinct AR-mediated effects on glucocorticoid regulation thus underlie the reduced GR-regulated transcriptional activity.

Sexual dimorphism exists in metabolic processes and affects incidence of metabolic disease. For example, expression of metabolic gene expression is highly divergent between male and female mice, particularly in the liver (Yang, et al. 2006). Although short-term fasting does not provoke many sex-specific effects in metabolic organs like WAT and muscle, the metabolic profile in the liver is greatly different between sexes (Della Torre, et al. 2018). This may in part be explained by differential exposure to sex hormones like androgens and estrogens. A direct role of androgens in the development of human cardiometabolic disease seems likely as this is more common in males versus females (Haring, et al. 2012; Yang and Kozloski 2011). Also female-to-male transgenders - in whom estrogens are replaced with androgens - present with increased body mass index and unfavorable lipid profiles (Quiros, et al. 2015; Velho, et al. 2017).

We can speculate that GR-AR interactions may contribute to metabolic dysfunction associated with endocrine conditions like Cushing's syndrome and polycystic ovary syndrome (PCOS). In PCOS patients, who are characterized by hyperandrogenism, metabolic abnormalities are remarkably

similar to those upon excess glucocorticoid exposure (e.g. adiposity, insulin resistance) (van Houten and Visser 2014). In our current study, we exclusively used male mice. Further studies in PCOS models in female mice (van Houten, et al. 2012) are warranted to fully outline the potential role of GR-AR interactions in this endocrine disease.

In our studies, most glucocorticoid-driven pathologies were found not to be androgen-dependent, with the notable exception of glucocorticoid-induced hyperlipidemia. It has to be noted however that Enzalutamide alone, independent of excessive CORT exposure, already lowered plasma lipid levels. Also, Enzalutamide treatment resulted in increased testosterone levels, which via aromatase can be converted to estradiol and provide metabolic benefit (Ohlsson, et al. 2017). Possibly a protective role of estrogens, rather than a harmful role of androgens, may thus contribute to the sexually dimorphic effects and cardiometabolic incidence. In support of this notion is that male and female mice treated with estrogens were protected from the complications of high-fat diet feeding (Dakin, et al. 2015; Kim, et al. 2014). In cancer, the estrogen receptor can also directly interact with the GR (West, et al. 2016), but it is unclear if a similar crosstalk is involved in metabolic GR signaling. Future research is thus warranted to fully establish the relative contributions of sex hormones – both androgen and estrogens – in glucocorticoid signaling and glucocorticoid-associated metabolic disease.

## Conclusion

We show crosstalk between glucocorticoid and androgen signaling in metabolic tissues (Fig. 8). These potentially bidirectional interactions can dramatically differ between tissues, as we found distinct patterns of nuclear receptor cooperation in the three different metabolic tissues that were analyzed. Our novel findings provide a proof-of-concept that interactions between different nuclear

receptor pathways exist in metabolic tissues. Further research is warranted to outline the relative contribution of glucocorticoid-androgen crosstalk to metabolic health.

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#### **Author contributions**

Conception and design: OM, JK

Acquisition of data: DS, MN, JB, HS, MS, EK, EB, NH, JK

Writing and review of manuscript: DS, MN, SK, PR, BW, OM, JK

Study supervision: BW, OM, JK

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**Figure legends****Figure 1: Confirmation of glucocorticoid and androgen interventions**

The effect of Enzalutamide on (A) Testosterone-induced and (B) DHT-induced TAT3 luciferase-reporter signaling, and on (C) basal and cortisol-induced GR signaling in human HEK293T cells. Data are mean±SEM and experiments were performed in quadruplicate. Statistical significance was calculated using an one-way analysis of variance. \*\*\*  $p < 0.001$  vs Veh, \$\$\$  $p < 0.001$  vs Test. or DHT. (D) Glucocorticoid-responsive adrenals and (E) androgen-responsive seminal vesicle organ weight in response to glucocorticoid and androgen interventions. Data are mean±SEM and N=6-7 per group. Statistical significance was calculated using a one-way analysis of variance with the Tukey multiple-comparisons test. \*\*  $p < 0.01$  vs Vehicle, \*\*\*  $p < 0.001$  vs Vehicle, \$\$\$  $p < 0.001$  vs CORT. (F-H) Dihydrotestosterone (DHT) and (I-K) Testosterone concentrations in plasma, WAT and liver. Data are mean±SEM and N=6-7 per group. Statistical significance was calculated using a one-way analysis of variance with the Tukey multiple-comparisons test. \*  $p < 0.05$  vs Vehicle, \*\*\*  $p < 0.001$  vs Vehicle, \$  $p < 0.05$  vs CORT, \$\$\$  $p < 0.001$  vs CORT.

**Figure 2: AR antagonism lowers glucocorticoid-induced hyperlipidemia**

(A) Body weight and composition and (B) plasma lipid levels of male C57BL6/J mice upon glucocorticoid and androgen interventions. Data are mean±SEM and N=6-7 per group. To calculate the change in body weight, fat and lean mass, measurements at day 14 and baseline values were used. Statistical significance was calculated using a one-way analysis of variance with the Tukey multiple-comparisons test. \*  $p < 0.05$  vs Vehicle, \*\*  $p < 0.01$  vs Vehicle, \*\*\*  $p < 0.001$  vs Vehicle, \$  $p < 0.05$  vs CORT, \$\$  $p < 0.01$  vs CORT, \$\$\$  $p < 0.001$  vs CORT. (C) Body weight and composition and (D) plasma lipid levels of male C57BL6/J mice upon Enzalutamide treatment. Data are mean±SEM and N=6 per group. To calculate the change in body weight, fat and lean mass, measurements at day 27

and baseline values were used. Statistical significance was calculated using a unpaired students t-test. \*\* p<0.01 vs Vehicle, \*\*\* p<0.001 vs Vehicle.

**Figure 3: AR signaling regulates GR-regulated transcriptional activity in metabolic tissues**

(A-C) WAT, (D-F) liver, (G-I) and BAT mRNA expression of GR-responsive genes *Fkbp5*, *Gilz* and *Mt2a*. Data are mean±SEM and N=6-7 per group. Statistical significance was calculated using a one-way analysis of variance with the Tukey multiple-comparisons test. \* p<0.05 vs Vehicle, \*\* p<0.01 vs Vehicle, \*\*\* p<0.001 vs Vehicle, \$ p<0.05 vs CORT, \$\$ p<0.01 vs CORT. (J) WAT and (K) liver FKBP5 protein expression. Representative Western images are shown and quantification of protein expression is based on N=6 per group. Data are mean±SEM and statistical significance was calculated using a one-way analysis of variance with the Tukey multiple-comparisons test. \* p<0.05 vs Vehicle, \*\* p<0.01 vs Vehicle, \*\*\* p<0.001 vs Vehicle, \$ p<0.05 vs CORT. (L) Expression of genes involved in WAT lipolysis, liver VLDL-production and BAT thermogenesis. \* p<0.05 vs Vehicle, \$ p<0.05 vs CORT. (M-N) The effect of Enzalutamide treatment *in vivo* on WAT and liver gene expression. Statistical significance was calculated by unpaired students t-test.

**Figure 4: AR signaling potentiates GR-regulated transcriptional activity in murine white and brown adipocytes**

(A-B) *Nr3c1* and *Nr3c4* expression in murine 3T3.L1 white adipocytes and cultured brown adipocytes. Data are mean±SEM and N=3 per group. (C-F) Murine 3T3.L1 white adipocyte mRNA expression of GR-responsive genes *Fkbp5*, *Gilz* and *Mt2a* and lipolysis gene *Atgl* upon exposure to 10-30 nM corticosterone and 100 nM dihydrotestosterone (DHT). (G-J) Brown adipocyte mRNA expression of GR-responsive genes *Fkbp5*, *Gilz* and *Mt2a* and thermogenic gene *Ucp1* upon exposure to 10-30 nM corticosterone and 100 nM dihydrotestosterone (DHT). Data are mean±SEM and experiments were performed in quadruplicate or quintuplicate. Statistical analysis was calculated with an unpaired

students t-test for each CORT dose comparing with and without DHT treatment. \*  $p < 0.05$  vs Vehicle, \*\*  $p < 0.01$  vs Vehicle, \*\*\*  $p < 0.001$  vs Vehicle.

**Fig 5: GR expression in WAT and liver**

(A-D) WAT and liver *Nr3c1* mRNA expression and GR protein expression upon glucocorticoid and androgen interventions. For RT-PCR data are mean $\pm$ SEM and N=6-7 per group. Statistical significance was calculated using a one-way analysis of variance with the Tukey multiple-comparisons test. Representative Western images are shown and quantification of protein expression is based on N=6 per group. GAPDH loading controls are identical to the ones shown in Fig. 3J-K. Statistical significance was calculated using a one-way analysis of variance with the Tukey multiple-comparisons test. \*\*  $p < 0.01$  vs Vehicle, \*\*\*  $p < 0.001$  vs Vehicle.

**Figure 6: AR signaling modulates systemic and tissue-specific glucocorticoid turnover**

(A-C) Concentrations of corticosterone (CORT) and (D-F) 11-dehydrocorticosterone (11-DHC), and (G-I) the ratio between CORT/11-DHC in plasma, WAT and liver. Data are mean $\pm$ SEM and N=6-7 per group. Statistical significance was calculated using a one-way analysis of variance with the Tukey multiple-comparisons test. \*  $p < 0.05$  vs Vehicle, \*\*  $p < 0.01$  vs Vehicle, \*\*\*  $p < 0.001$  vs Vehicle, \$  $p < 0.05$  vs CORT, \$\$  $p < 0.01$  vs CORT.

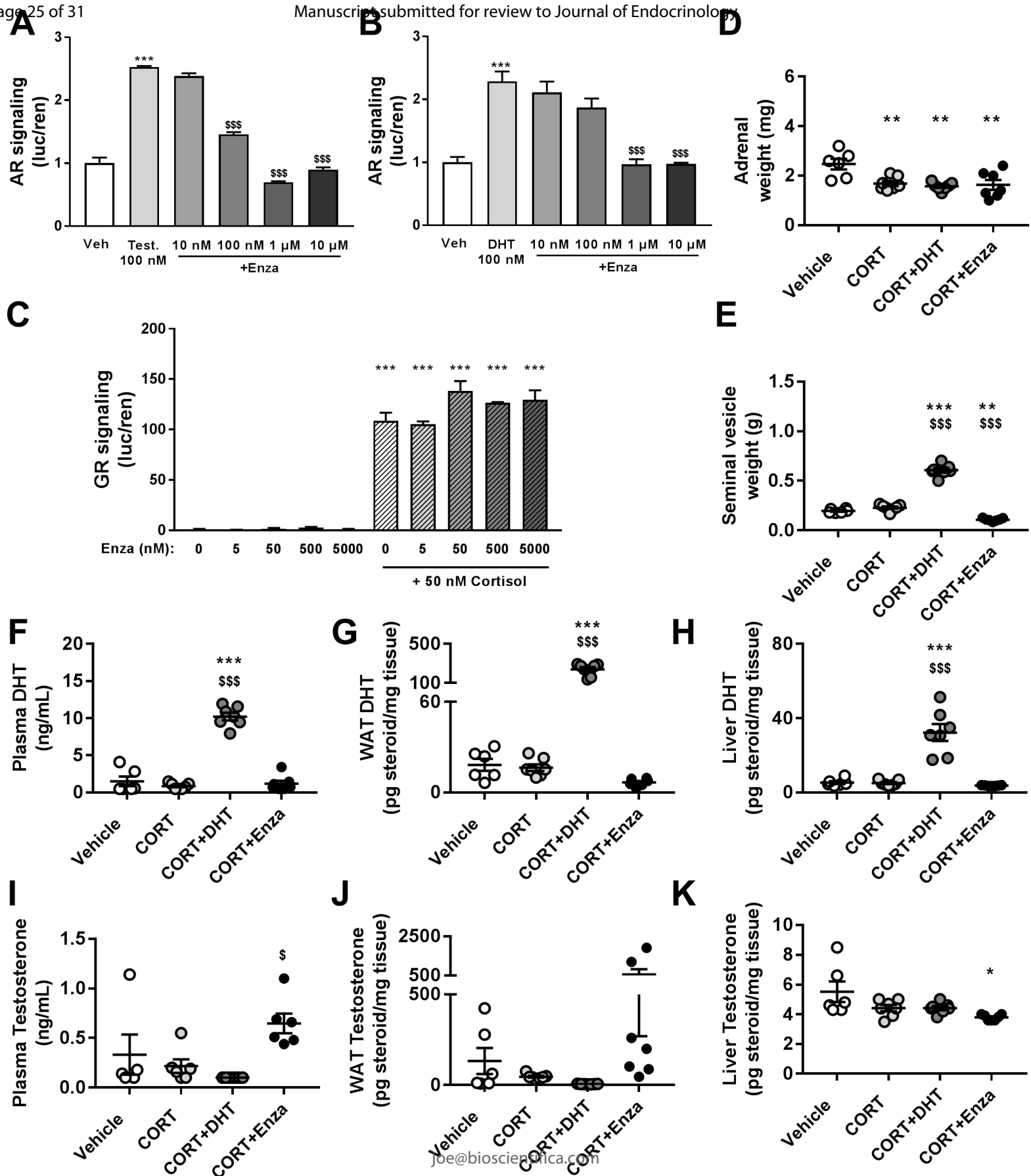
**Figure 7: AR signaling modulates 11BHSD1 and contributes to GR-regulated transcriptional activity in WAT but not liver**

(A-D) *Hsd11b1* mRNA and 11BHSD1 protein expression in WAT and liver upon glucocorticoid and androgen interventions. (E-F) The relationship between CORT levels and the expression of GR-responsive genes *Fkbp5*, *Gilz* and *Mt2a* in WAT and liver. Data are mean $\pm$ SEM and N=6-7 per group. Statistical significance was calculated using a one-way analysis of variance with the Tukey multiple-comparisons test. \*  $p < 0.05$  vs Vehicle, \*\*  $p < 0.01$  vs Vehicle, \$  $p < 0.05$  vs CORT, \$\$  $p < 0.01$  vs CORT.

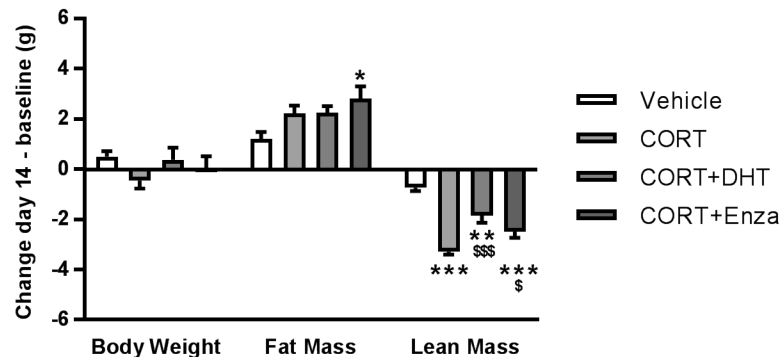
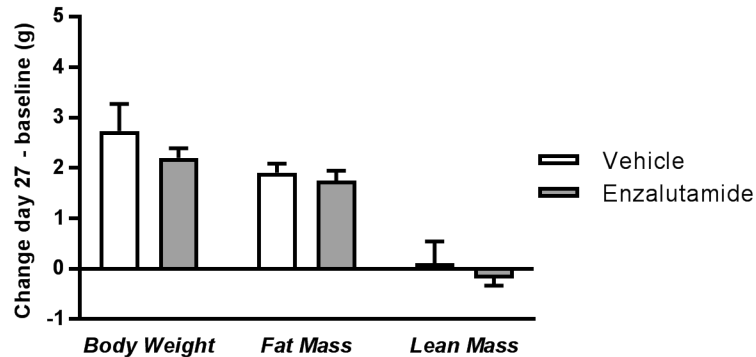
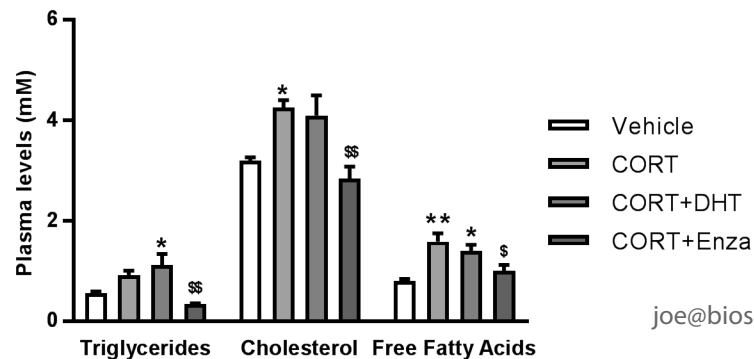
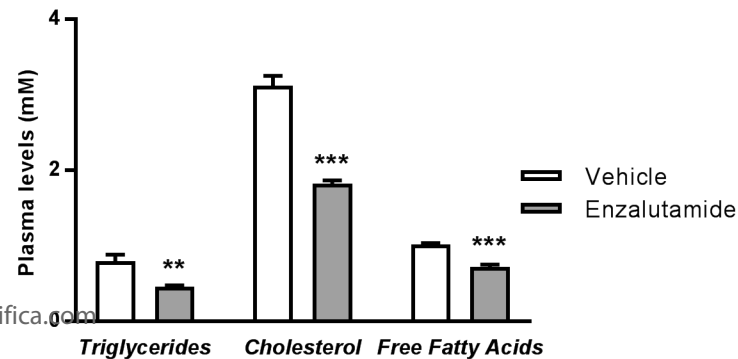
561 Representative Western images are shown and quantification of protein expression is based on 6  
562 per group. GAPDH loading controls in Fig. 7D are identical to the ones shown in Fig. 3K.

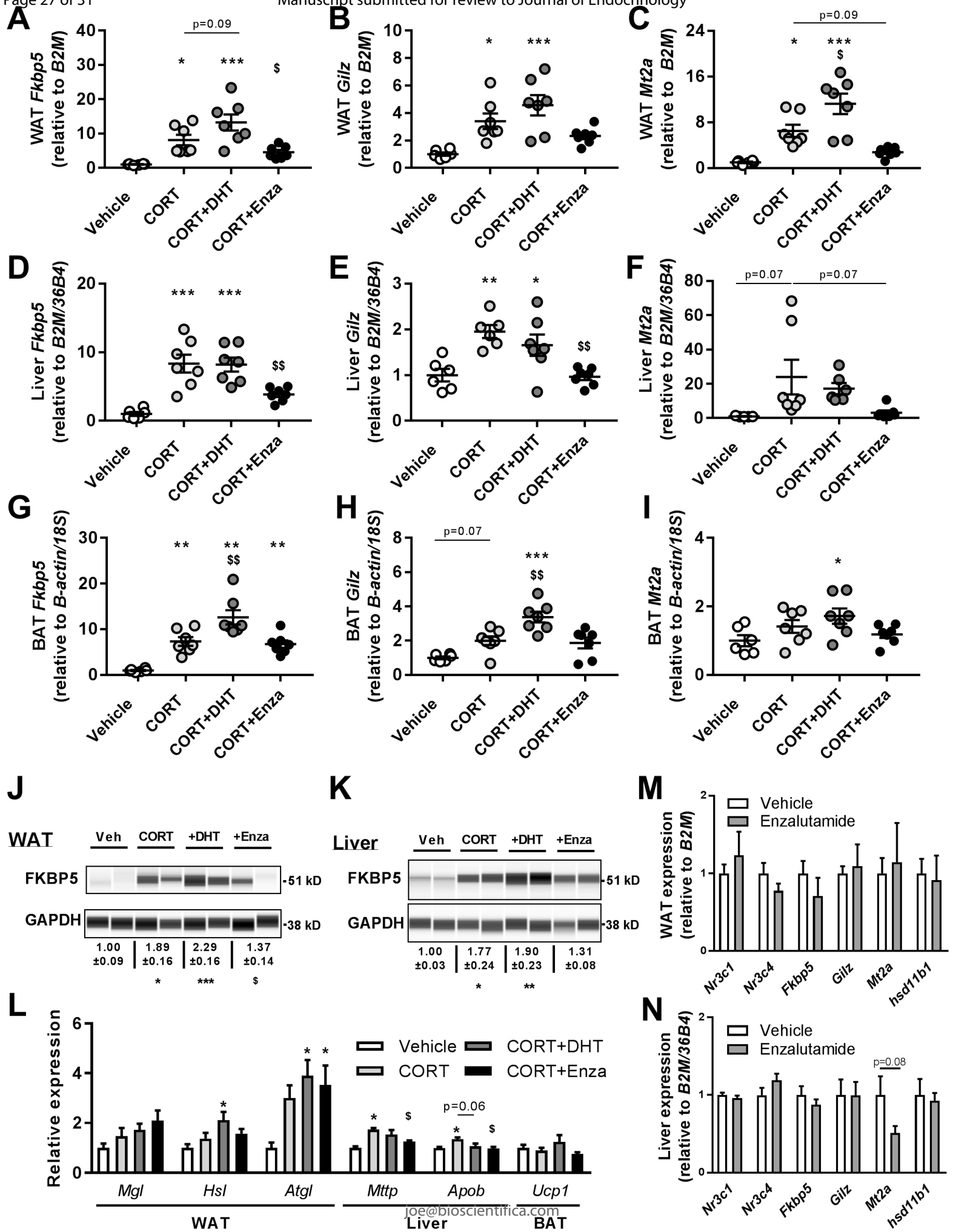
563 **Figure 8: Schematic overview of glucocorticoid-androgen crosstalk in WAT, liver and BAT.**

For Review Only

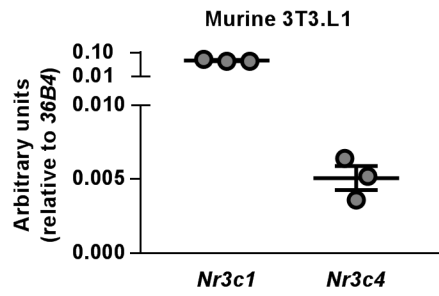




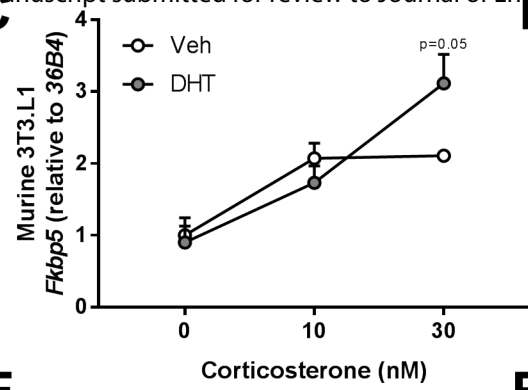
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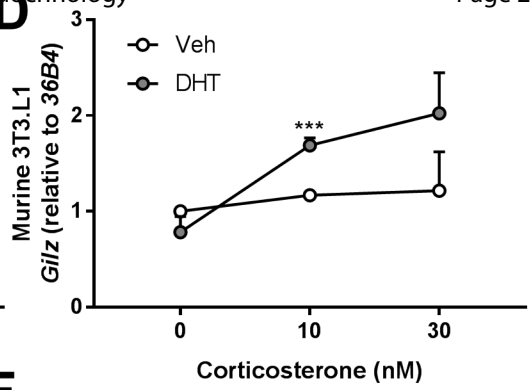
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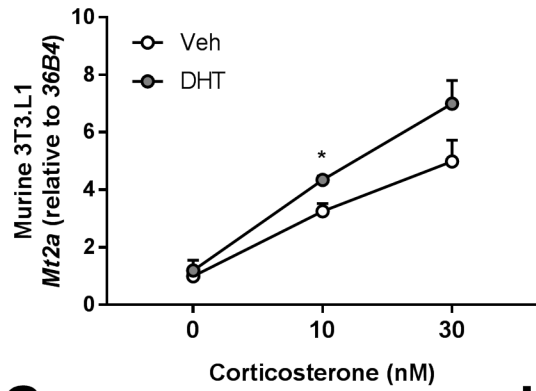
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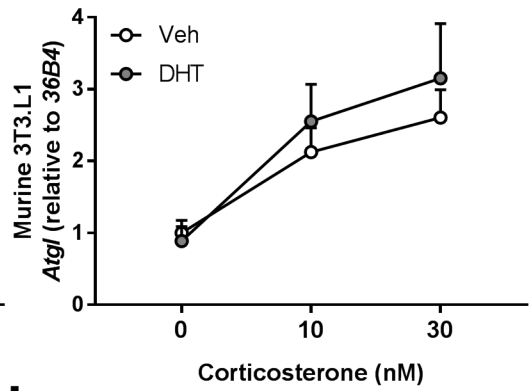
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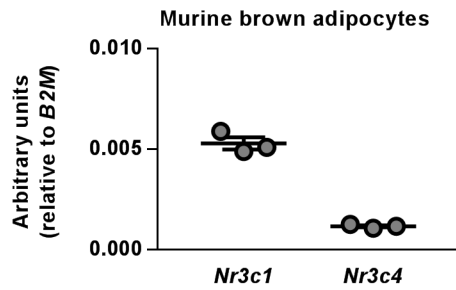
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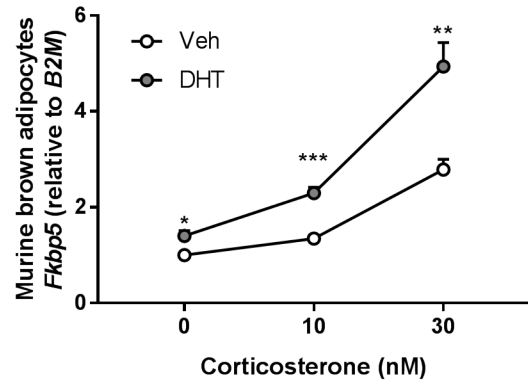
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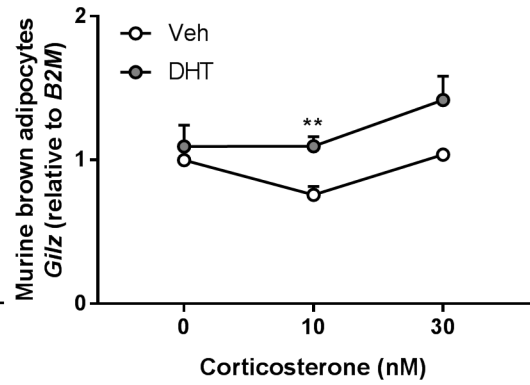
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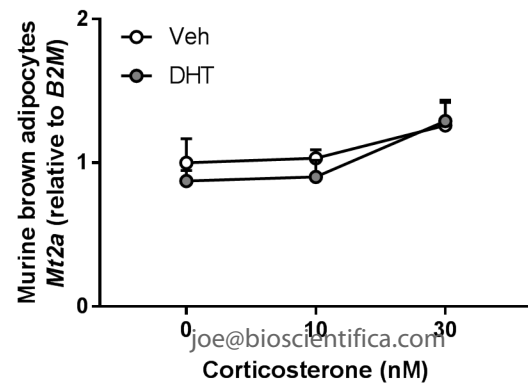
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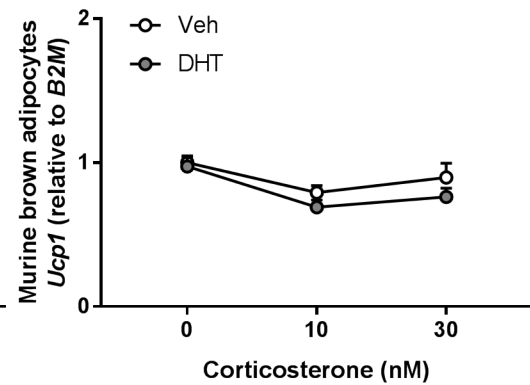
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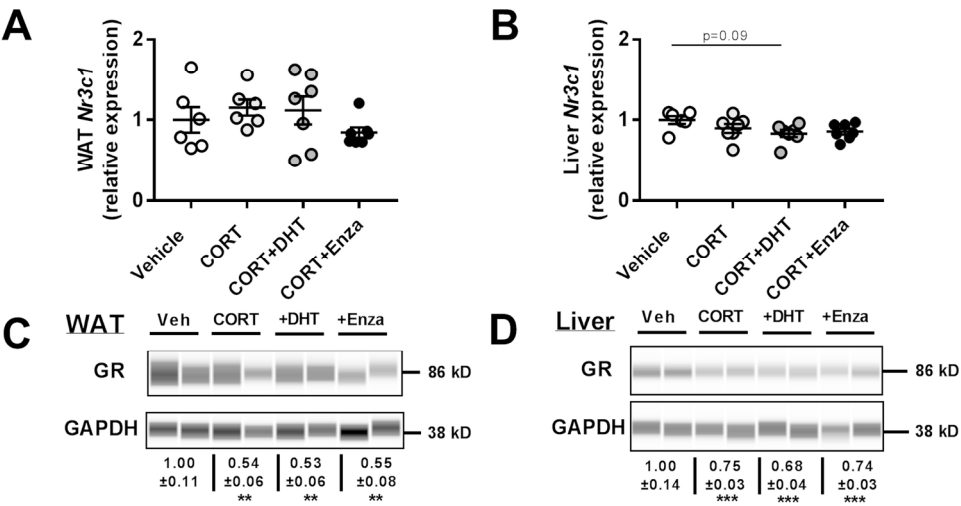
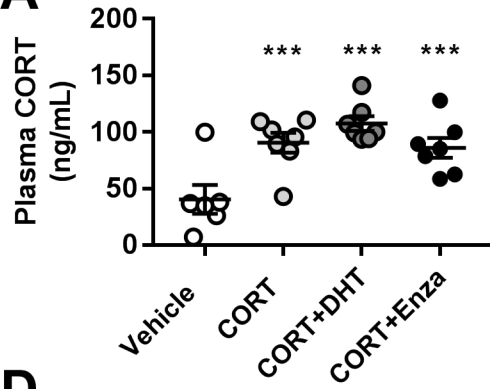
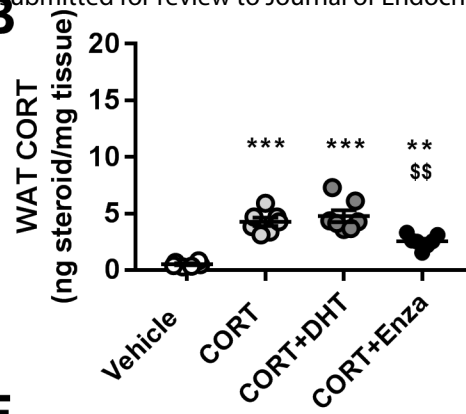
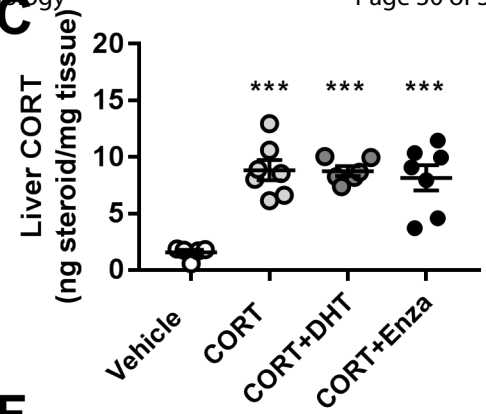
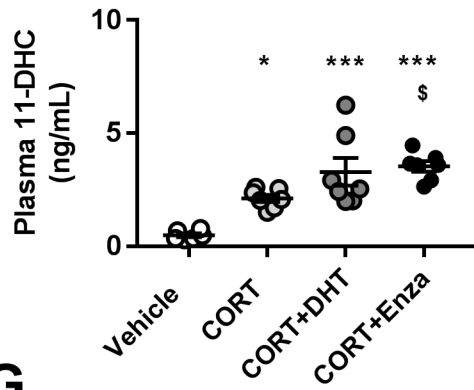
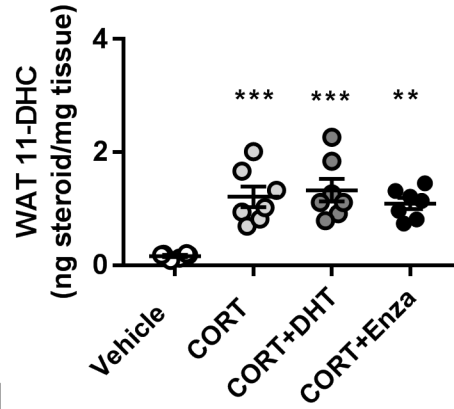
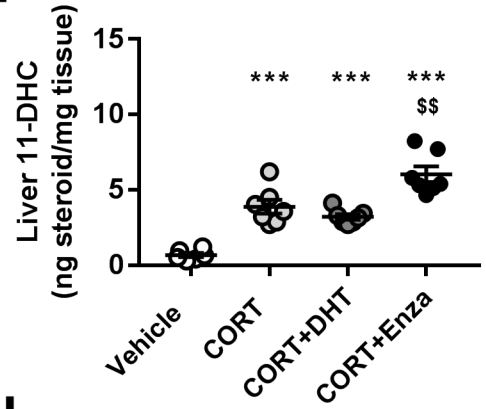
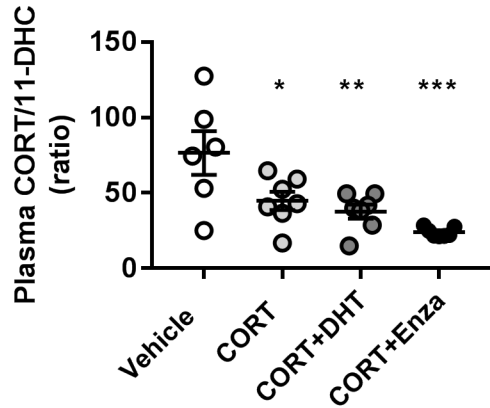
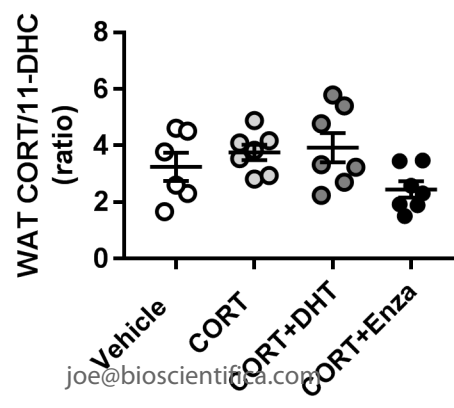
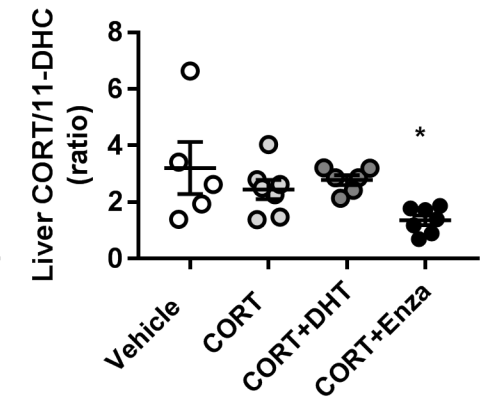
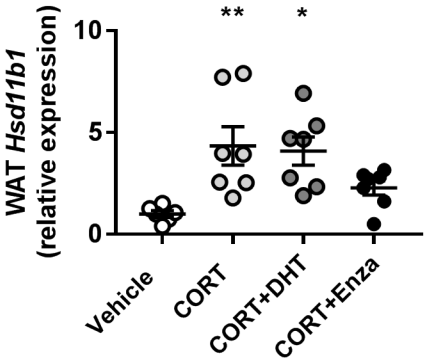


Fig 5: GR expression in WAT and liver

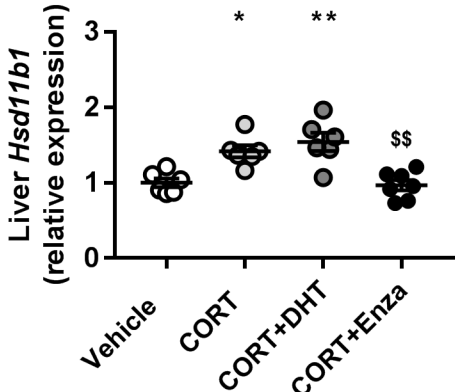
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**A****B****C****D****E****F****G****H****I**

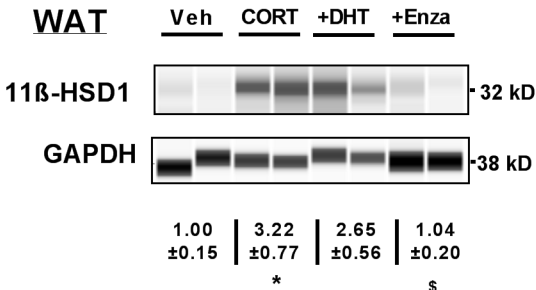
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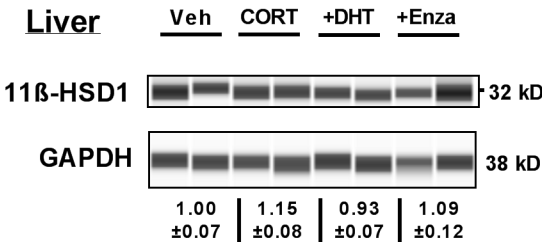
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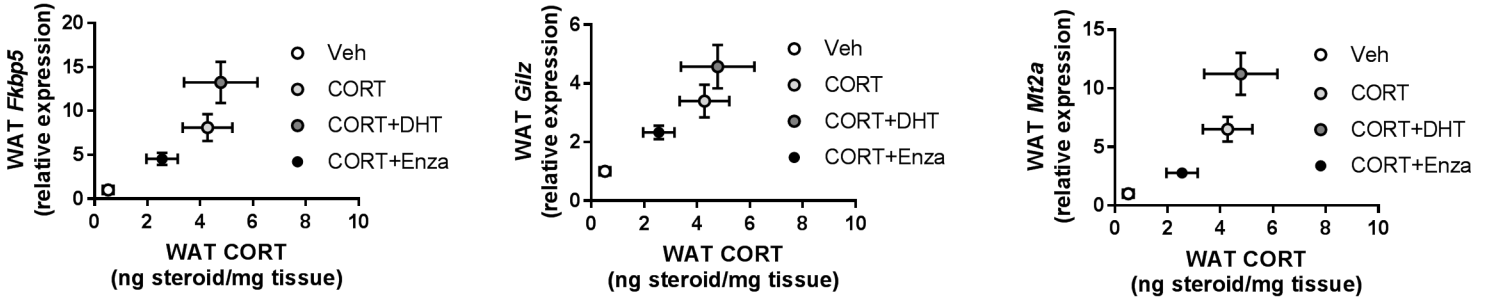
**C**



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